Mechanisms of Cell Injury by Activated Oxygen Species

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Current evidence suggests that O_2^- and H_2O_2 injure cells as a result of the generation of a more potent oxidizing species. In addition to O_2^- and H_2O_2, the third essential component of the complex that mediates the lethal cell injury is a cellular source of ferric iron. The hypothesis most consistent with all the available data suggests that O_2^- reduces a cellular source of ferric to ferrous iron, and the latter then reacts with H_2O_2 to produce a more potent oxidizing species, like the •OH or an equivalently reactive species. In turn, •OH initiates the peroxidative decomposition of the phospholipids of cellular membranes. •OH also damages the inner mitochondrial membrane. Upon mitochondrial deenergization, a sequence of events is initiated that similarly leads to the loss of viability of the cell. DNA represents a third cellular target of •OH. Depending on the cell type, oxidative DNA damage can be coupled to cell killing through a mechanism related to the activation of poly (ADP-ribose) polymerase. — Environ Health Perspect 102(Suppl 10):17–24 (1994)

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Introduction

The reduction of molecular oxygen to water by the addition of four electrons is the major source of energy for most aerobic organisms. This reaction, strongly favored thermodynamically, yields 105.2 kcal/mole of O_2 reduced to H_2O. Much of this energy is not lost, but is stored as a chemiosmotic gradient across the inner mitochondrial membrane. Neither molecular oxygen, because of its two unpaired electrons of similar spin, nor water reacts very readily with biologic molecules. However, partially reduced and more reactive oxygen molecules can be formed under a variety of circumstances that are associated with disease in humans. Such activated oxygen species are increasingly recognized to be the mediator of the cell injury in these diseases.

Partially Reduced Oxygen Species: Formation and Catabolism

The addition of one electron to dioxygen (O_2) yields the superoxide anion (O_2^-). O_2^- is formed nonenzymatically in virtually all aerobic cells as a result of the autooxidation of the constituents of the mitochondrial electron transport chain. In addition, O_2^- is generated enzymatically by a number of oxidases, other than cytochrome oxidase, which include xanthine oxidase and the oxidase present in the plasma membrane of the phagocytic cells of acute and chronic inflammation. Two molecules of superoxide anion (O_2^-) react simultaneously, or enzymatically as a consequence of the activity of SOD, to form dioxygen and hydrogen peroxide (H_2O_2). Thus, H_2O_2 is almost always formed under any circumstance where there is the generation of O_2^-. Superoxide dismutases are found in the mitochondria and in the cytosol, a localization that agrees with sites of generation of O_2^-.

H_2O_2 is also formed by the direct two electron reduction of O_2, a reaction that is catalyzed by a number of oxidases generally found in eukaryotic cells in peroxisomes, membrane-bound cytoplasmic organelles. H_2O_2 is reduced to water by two different enzymes: catalase in peroxisomes, and glutathione peroxidase present in mitochondria and in the cytosol.

The oxygen–oxygen bond of H_2O_2 can be split by the addition of one electron, a reaction that yields the relatively innocuous hydroxide anion (HO^-) and the highly reactive hydroxyl radical (•OH). The best known sources of electrons for the reduction of H_2O_2 are transition metal cations, particularly ferrous iron and the cuprous ion. Ferrous iron reduces H_2O_2 to form •OH and a hydroxyl anion with the accompanying formation of ferric iron, a chemistry known as the Fenton reaction. The only other potential source of •OH in biologic systems is the radiolysis of water by ionizing radiation. Importantly, such a reaction does not require the participation of a transition metal cation.

Cell and Tissue Injury by Activated Oxygen Species

All aerobic cells generate, enzymatically or nonenzymatically, a constitutive flux of O_2^-, H_2O_2, and possibly •OH. At the same time, the abundant antioxidant defenses of most cells, again both enzymatic and nonenzymatic, prevent these species from causing cell injury. Nevertheless, there are situations in which the rate of formation of partially reduced oxygen species is increased and/or the antioxidant defenses of the cells are weakened. In either case, oxidative cell injury may result.

By increasing the oxygen tension in the environment of cells, most notably in the pulmonary alveoli through therapeutically elevating the concentration of oxygen in the inspired air, the rate of autooxidation of mitochondrial electron carriers is increased. Thus, the rate of formation of O_2^- and H_2O_2 is higher with an increased oxygen tension. Such chemistry is the likely basis of the pulmonary injury of oxygen toxicity.

During the process of phagocytosis, inflammatory cells generate O_2^- and, by dismutation of these ions, H_2O_2. The cell and tissue injury associated with acute and chronic inflammation is attributable, at least in part, to the toxicity of these activated oxygen species.

The metabolism of many drugs and other xenobiotic chemicals produces both O_2^- and H_2O_2. Evidence continues to accumulate that the toxicity of such chemicals is mediated by oxidative stress (*1*), rather

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than, as has been held for many years, by the cellular interactions of the reactive metabolites derived from the parent compounds.

Finally, considerable attention in the last few years has been directed to the phenomenon of reperfusion injury. In this situation, ischemic tissues are not irreversibly injured during the period of anoxia. Rather, they are injured after reperfusion, as a result of the formation of activated oxygen species either by inflammatory cells, such as the neutrophil (*2*), or by the activity of intracellular enzymes, such as xanthine oxidase (*3*).

The Fate of Hydrogen Peroxide: Two General Mechanisms of Oxidative Cell Injury

Two hypotheses have been invoked to explain the mechanisms of cell injury by partially reduced, and thereby activated, oxygen species. These hypotheses differ in their interpretation of the consequences of the catabolism of H_2O_2: one views glutathione peroxidase as a detoxification pathway; the alternative views it as a toxification pathway. As noted above, an increased formation of H_2O_2 can occur directly or as a consequence of any situation where there is an increased formation of O_2^-. The only exception to the central role of hydrogen peroxide in the toxicity of activated oxygen species is the direct formation of the •OH as a result of the radiolysis of water. Thus, the mechanism of cell injury by ionizing radiation is treated as a special case, when it is argued that glutathione peroxidase is a toxifying activity, or as a corollary of the argument that this pathway is indeed detoxifying.

By the addition of two electrons, glutathione peroxidase reduces H_2O_2 to water. At the same time, reduced glutathione (GSH) is oxidized to oxidized glutathione (GSSG). GSSG is reduced to GSH by glutathione reductase. With this latter reaction, NADPH is oxidized to NADP. Thus, increased glutathione peroxidase activity shifts the major, soluble redox active cofactors from a predominantly reduced state to a predominantly oxidized state, GSH to GSSG and NAD(P)H to NAD(P). In addition, an increase in GSSG is paralleled by an increase in oxidized protein-bound thiols, largely because GSSG can react with protein-bound thiols to form glutathione mixed disulfides.

These changes in both soluble and protein-bound thiols, and the change in pyridine nucleotide metabolism, are accompanied by a release into the cytosol of sequestered stores of calcium (*4,5*). As a consequence, there is a rise in the cytosolic concentration of free calcium $[Ca^{2+}]_i$ (*6*). It is hypothesized that this rise in $[Ca^{2+}]_i$ mediates the cell injury associated with an acute oxidative stress (*7,8*).

The alternative hypothesis holds that the critical reaction in the initiation of cell injury by H_2O_2 is not its catabolism by glutathione peroxidase, but rather the formation of a more potent oxidizing species, such as the •OH, generally by a Fenton reaction with ferrous iron or another transition metal cation, such as cuprous ions (*9*). According to this hypothesis, glutathione peroxidase is indeed a detoxification activity, because it removes H_2O_2 and thus prevents the formation of a more potent oxidizing species. The changes in thiol, pyridine nucleotide, and calcium homeostasis that occur with the catabolism of H_2O_2 by glutathione peroxidase are considered simply epiphenomena, rather than critical events in the toxicity of H_2O_2.

The interaction of the •OH, or an equivalently reactive species, with a number of cellular targets initiates those events that lead to the eventual death of the cell. The most important of such targets include *a*) the phospholipids of cellular membranes with the initiation and propagation of lipid peroxidation, *b*) the mitochondrial inner membrane with a loss of energization and cellular stores of ATP, and *c*) the DNA with the appearance of single-strand breaks and their subsequent repair.

Glutathione Peroxidase as a Toxifying Pathway: The Role of Alterations in Intracellular Calcium Homeostasis

The loss of viability with oxidative cell injury is proposed by one hypothesis to be a consequence of the sequence of events that proceeds from alterations in glutathione metabolism to elevated $[Ca^{2+}]_i$. This hypothesis is based on experimental data of three sorts.

First, exposure of suspensions of isolated rat hepatocytes to *t*-butyl hydroperoxide (TBH), a stable substrate for glutathione peroxidase, or to the H_2O_2 generated by the redox cycling of the quinone derivative menadione, impaired the sequestration of calcium by mitochondria and the endoplasmic reticulum (*4,5*). There was an accompanying rise in $[Ca^{2+}]_i$ (*6*). Altered intracellular calcium homeostasis was attributed to the depletion of glutathione and the oxidation of pyridine nucleotides that accompanied the reduction of GSSG by glutathione reductase. Blebbing of the surface membrane followed that alteration in calcium homeostasis and reflected changes in the cytoskeleton (*4,5,10*).

Second, conditions that protected against the cell injury, notably the presence of the sulfhydryl reagent dithiothreitol, also prevented the increase in $[Ca^{2+}]_i$ and the loss of Ca^{2+} from its intracellular storage pools (*4*). Third, direct mobilization of calcium from these stores by the ionophore A23187 similarly promoted cell killing that was preceded by an elevated $[Ca^{2+}]_i$ (*4*).

Initially, calcium release was felt to occur from both the endoplasmic reticulum and mitochondria, the former in relation to the depletion of GSH and the latter in response to the oxidation of NADPH (*4*). However, mitochondria do not normally sequester significant stores of calcium (*11*). Thus, alterations in the sequestration of calcium by the endoplasmic reticulum alone coupled with an inhibition of the calcium extrusion pump of the plasma membrane (*6,12,13*) are the major mechanisms responsible for the elevated $[Ca^{2+}]_i$. This disturbed calcium homeostasis has, in turn, been related to the loss of protein thiol groups (*14,15*), a change considered to be the critical event in the genesis of lethal cell injury with an oxidative stress. In other words, the loss of protein thiols in both the plasma membrane and endoplasmic reticulum disrupts the function of proteins critical to the regulation of calcium homeostasis.

Three mechanisms have been invoked to account for the loss of protein thiol groups (*14*). First, protein thiols are lost as a result of their reaction with GSSG to form glutathione mixed disulfides (*14*). Second, a loss of protein thiols follows their direct oxidation to disulfides, presumably by partially reduced oxygen species (*14*). Third, in the case of the toxicity of menadione, protein thiols are lost as a result of their arylation by the quinone itself (*14*).

Several observations are used to argue that changes in protein thiols are linked to a loss of viability. A depletion of protein thiols preceded the loss of viability of suspensions of hepatocytes exposed to the oxidative stress produced by the metabolism of toxic quinones (*15,16*). In turn, conditions that accelerated the loss of protein thiols sensitized the cells to the toxicity of these quinones (*16*). By contrast, sulfhydryl reagents prevented the loss of protein thiols and prevented the loss of viability (*15*). Similarly, addition of vitamin E to suspensions of calcium-depleted hepatocytes maintained protein thiol levels in parallel with protection against the cell killing (*16*).

In suspensions of isolated hepatocytes, cystamine reacted directly with protein thiols to form mixed disulfides with a consequent inhibition of a plasma membrane

Ca^{2+}-ATPase activity and a decreased rate of Ca^{2+} efflux from the cells (*17*). The resulting intracellular accumulation of Ca^{2+} was followed by a stimulation of both phospholipid hydrolysis and proteolysis (*17*). Interestingly, pretreatment of the cell with protease inhibitors protected the hepatocytes from the toxicity of cystamine, whereas phospholipase inhibitors did not (*17*).

Alterations in intracellular calcium homeostasis attendant on the depletion of both glutathione and protein thiol groups provide a consistent hypothesis of the sequence of events that mediate the lethal cell injury from an oxidative stress. Release of calcium stores certainly occurs upon the reduction of H_2O_2 and the accumulation of GSSG as a result of glutathione peroxidase activity. Furthermore, disruption of intracellular calcium homeostasis by treatment of a variety of cell types with the calcium ionophore A23187 can precipitate cell death. Finally, activation of nonlysosomal proteases, in addition to other degradative processes, is a reasonable explanation of the mechanism by which an elevated $[Ca^{2+}]_i$ mediates lethal cell injury. However, there are problems with this hypothesis.

The most serious deficiency of this hypothesis is that it ignores alternative pathways for the metabolism of H_2O_2. In particular, it ignores the iron-dependent formation of more potent oxidizing species from H_2O_2. When the formation of such oxidizing species is prevented, or the toxic effects of their interactions with cellular constituents is prevented, there is no effect on the biochemical consequences of the metabolism of H_2O_2 by glutathione peroxidase: GSH is lost with the parallel accumulation of GSSG; protein thiols are depleted, and there is an increase in the cytosolic calcium ion concentration. However, despite these changes, the cells do not die.

In this manner, the killing of cultured hepatocytes by either H_2O_2 or TBH has been dissociated from changes in intracellular calcium homeostasis (*18–20*). The lethal cell injury after exposure of these cells to either of the peroxides depended on the formation of ·OH or *t*-butyl alkoxyl radicals by Fenton chemistry (*18,9*). Formation of these radials was prevented and the liver cells protected from the toxicity of either H_2O_2 or TBH by chelating a cellular pool of ferric iron with deferoxamine (*18,9*). However, hepatocytes pretreated with deferoxamine still manifested the same disordered calcium homeostasis as occurred in nondeferoxamine-treated cells (*18–20*).

The radicals formed in the reaction of H_2O_2 or TBH with iron initiated the peroxidative decomposition of membrane phospholipids of cultured hepatocytes (*18,21*). Antioxidants added to the culture medium prevented both this lipid peroxidation and the death of the cells (*18,21*) without any effect on the reduction of either peroxide by glutathione peroxidase (*18*).

Thus, changes in calcium homeostasis occur without accompanying irreversible liver cell injury from an oxidative stress (*18–20*). Conversely, irreversible injury can occur without changes in calcium homeostasis (*19*). Pretreating cultured hepatocytes with EGTA in a calcium-free medium removed over 75% of the cell-associated calcium, lowered the basal $[Ca^{2+}]_i$, and eliminated its rise in response to H_2O_2. However, these same cells were not resistant to the toxicity of H_2O_2 (*19*).

Similar to the disruption of calcium homeostasis, changes in protein thiols can be dissociated from the cell killing by an oxidative stress (*22,23*). Three mechanisms have been identified by which protein thiols are lost with the oxidative killing of cultured hepatocytes (*22*). Two of these mechanisms, namely formation of mixed disulfides and arylation of protein thiols, were observed to deplete protein thiols upon exposure to menadione (*22*). However, reduction of protein thiols by these two mechanisms did not correlate with the extent of cell killing (*22*).

Metabolism of acetaminophen by cultured rat hepatocytes depleted protein thiols as a result of the formation of glutathione-mixed disulfides and by arylation (*23*). However, in the presence of deferoxamine, there was little or no cell killing for up to 8 hr despite a loss of 60% of the total protein thiols (*23*).

In addition to the formation of glutathione-mixed disulfides, protein thiols were lost as a result of the peroxidation of membrane lipids during the intoxication of cultured hepatocytes with the H_2O_2 generated in the cultured medium by glucose oxidases (*22*). However, it is likely that this loss is simply an epiphenomenon of the direct destruction of membrane integrity by the peroxidation of unsaturated fatty acids.

A second problem with the hypothesis that attributes oxidative cell injury to alterations in calcium homeostasis is its derivation from studies that almost exclusively used suspensions of freshly isolated hepatocytes exposed to an atmosphere of 95% O_2–5% CO_2. When compared with hepatocytes cultured for 24 hr with room air (20% O_2), freshly suspended cells are more fragile and are particularly sensitive to both the presence or absence (*24–27*) of extracellular calcium ions. In other words, the experimental model used to generate the data implicating an alteration in calcium homeostasis manifests exaggerated calcium responses to a variety of toxic injuries. Such responses are not representative of the reaction of liver cells to the same hazards, when these cells are studied under more physiologically relevant conditions.

The antioxidant catechol prevented the killing by TBH of freshly suspended hepatocytes (*28*). However, under such circumstances and in contrast to the results with cultured hepatocytes (*18*), the reduced cell killing was accompanied by a lower $[Ca^{2+}]_i$ (*28*). Lipid peroxidation in the suspended hepatocytes likely rendered them permeable to extracellular calcium ions, an effect that would not occur until later times with cultured cells. An influx of extracellular calcium would further elevate $[Ca^{2+}]_i$, an effect that might also aggravate the underlying mechanism of irreversible injury. Nevertheless, protection by the antioxidant was not primarily a consequence of the dampened rise in cytosolic calcium. Cultured hepatocytes protected by the same antioxidant showed no such difference in cytosolic calcium compared to unprotected cells (*18*), a result that reflects the absence of the additional effect of an influx of extracellular calcium ions.

The mechanism of the killing of cultured hepatocytes by cystamine could not be attributed to an alteration in calcium homeostasis (*29*). The cytosolic calcium ion concentration in cultured hepatocytes did rise before the loss of viability (*29*). However, treatment with EGTA in Ca^{2+}-free medium prevented this rise again without effect on the toxicity of cystamine (*29*). Furthermore, the sensitivity of cultured hepatocytes to cystamine was unaffected by the concentration of calcium in the culture medium (*29*). Addition to the culture medium of three different protease inhibitors did not reduce the extent of cell killing by cystamine, despite an inhibition of protein degradation (*29*). An exaggerated sensitivity of suspensions of freshly isolated hepatocytes to extracellular calcium readily explains the differing effects of cystamine in the two models. By inhibiting the active extrusion of calcium from the suspended hepatocytes, cystamine allowed an accelerated influx of calcium to produce an exaggerated rise in $[Ca^{2+}]_i$ such

that protease activation occurred (*17*). In cultured hepatocytes, such a response does not occur, and cell killing is achieved by a mechanism unrelated to activation of proteases (*29*).

In summary, current evidence suggests that the alterations in intracellular calcium homeostasis that are attendant on the depletion of reduced glutathione and the loss of protein thiols are likely epiphenomena unrelated to the mechanism of lethal injury.

Glutathione Peroxidase as a Detoxifying Pathway: Formation from Hydrogen Peroxide of a More Potent Oxidant

The alternative hypothesis to altered calcium homeostasis argues that the toxicity of H_2O_2 is mediated by the formation of a more potent oxidizing species in reaction that requires iron. Both the exact nature of this oxidizing species and the source of the iron required for its formation have been a matter of considerable debate. Recent studies of the mechanisms of the killing of cultured hepatocytes by H_2O_2 or TBH have provided some resolution of these concerns.

As noted above, the killing of cultured rat hepatocytes by either H_2O_2 or TBH depends on a cellular source of ferric iron (*9,18*). This conclusion is based on the ability of deferoxamine, a ferric iron chelator, to prevent the cell killing by either toxin. Importantly, the hepatocytes can be pretreated with deferoxamine, washed thoroughly, and then exposed to the peroxide. This regimen allowed the conclusion that the action of deferoxamine was a consequence of the chelation of a cellular pool of iron, rather than an extracellular interaction with iron possibly contaminating the medium. Phenanthroline, another iron chelator, prevented the killing of mouse 3T3 cells by H_2O_2 (*30*).

The sensitivity of hepatocytes pretreated with deferoxamine to an oxidative stress can be restored by either of two ways. In one, the addition of either ferric or ferrous iron to the culture medium resulted in a prompt return of sensitivity (*31*). In the other, incubation of the deferoxamine-pretreated hepatocytes in a serum-free medium containing only 0.25 nM iron restored the sensitivity of the cells after 4 to 6 hr (*32*). Thus, the iron required for the killing of hepatocytes by an oxidative stress can be derived from the medium or by the mobilization of iron from intracellular stores.

In addition to ferric iron, O_2^- are required for expression of the toxicity of either H_2O_2 or TBH. This dependence on a cellular source of superoxide ions is indicated by the ability of SOD to prevent the toxicity of TBH (*33*) or the H_2O_2 that was generated in the medium by glucose oxidase (*9*). Glucose oxidase catalyzes the two-electron reduction of molecular oxygen to H_2O_2 without the intermediate formation of O_2^-. Thus, the O_2^- required for the cell killing by H_2O_2, and by TBH as well, are produced by the hepatocytes.

The uptake by endocytosis of SOD is required for the enzyme to protect the hepatocytes (*33,34*). Inhibitors of endocytosis abolish the protective effect of SOD at the same time that they abolish the increase in cell-associated SOD activity (*33,34*). Liposome-encapsulated SOD will also protect hepatocytes from the toxicity of TBH (*33*). Liposome-encapsulated SOD is more readily internalized by the hepatocytes, as considerably less liposome-encapsulated SOD is needed to protect the cells (*33*). In addition, uptake of the liposome-encapsulated SOD is not affected by the same inhibitors of endocytosis that prevent the uptake of the free enzyme (*33*).

H_2O_2, or TBH, reacts with ferrous iron to produce a more potent oxidant, the •OH or *t*-butyl alkoxyl radical (*9,18*). However, for H_2O_2 (or TBH) to kill cultured hepatocytes, the target cell must contribute a source of O_2^- and ferric iron. Thus, it is not immediately obvious what role is played by either O_2^- or ferric iron.

Ferric or ferrous iron restores the sensitivity of deferoxamine pretreated hepatocytes to H_2O_2 (*9*). SOD prevents the restoration of sensitivity by ferric, but not by ferrous iron (*9*). When the H_2O_2 is generated by the redox cycling of menadione, similar results are obtained (*9*). Thus, ferrous iron requires only H_2O_2 for restoration of sensitivity, whereas ferric iron requires O_2^- as well as H_2O_2.

A simple mechanism can explain these requirements. A cellular pool of ferric iron is reduced to ferrous iron by O_2^-. In turn, H_2O_2 is reduced by this ferrous iron to form •OH, the highly reactive species that actually mediates the lethal cell injury. Such a sequence is referred to as an iron-catalyzed Haber-Weiss reaction and is postulated to be the major mechanism whereby activated oxygen species lead to the irreversible cell injury of cultured hepatocytes (*9*).

Biology of Intracellular Iron Required for Oxidative Cell Injury

The above discussion leaves several issues unresolved, in particular, the nature of the cellular source of the iron required for cell killing, its location within the cell, and the mechanism that generates it.

Ferritin, the major iron storage protein in virtually all cells, is the likely source of the pool of ferric iron required for the cell killing by either H_2O_2 or TBH. Two mechanisms are proposed for the mobilization of iron from ferritin. It is still widely held that the release of iron from intact ferritin occurs upon the reduction of ferric to ferrous iron without a requirement for the degradation of the apoprotein. Under *in vitro* conditions, many potentially physiologic, and clearly nonphysiologic, reducing agents are capable of releasing iron from ferritin. However, definitive identification of the reductant(s) that operates within the intact cell has not been made.

It has been proposed that oxidative stress itself may reductively release iron from ferritin and thereby promote cell injury (*35–37*). O_2^- by xanthine oxidase or by stimulated polymorphonuclear leukocytes reductively released iron from ferritin (*6,36*). Furthermore, redox cycling of paraquat promoted iron release from ferritin by both oxygen-dependent and oxygen-independent mechanisms. Thus, the generation of superoxide anions (oxygen-dependent) and the paraquat-free radical itself (oxygen-independent) released iron from ferritin, a process suggested to contribute to the toxicity of such chemicals (*36,37*).

There are, however, problems with such an origin of the ferric iron required for cell injury by activated oxygen species. Whereas it readily accounts for the requirement for O_2^-, the hypothesis does not explain the requirement for deferoxamine-chelated ferric iron. Iron is stored in ferritin in the ferric state, but such ferric iron is largely inaccessible to deferoxamine. Thus, the pool of ferric iron removed by deferoxamine must be distinct from ferritin iron. Its biologic significance is not clarified by the suggestion that O_2^- may release ferrous iron from ferritin. Furthermore, hepatocytes are protected by treatment with deferoxamine before their exposure to a source of O_2^-. Thus the requisite pool of iron must have an origin independent of the oxidative stress.

Iron is also liberated from ferritin as a consequence of the intracellular degradation of this protein. Like that of many cellular proteins, the turnover of ferritin involves the organelles of the autophagic vacuolar apparatus. Intracellular ferritin is engulfed within autophagic vacuoles by the folding of the endoplasmic reticulum around portions of the cell sap. These vacuoles then

fuse with lysosomes to become autophagosomes, organelles within which the ferritin is degraded with the release of ferric iron.

Conditions that affect the autophagic degradation of proteins similarly affect the regeneration of the iron pool required to restore sensitivity of deferoxamine-pretreated hepatocytes to TBH (*32*). These conditions include both the activation of autophagic protein degradation by amino acid starvation a well as its inhibition by a variety of chemicals (*32*). A reductive release of iron from ferritin by O_2^- is unlikely to account for the restoration of sensitivity. The ability to inhibit or enhance this process cannot be explained by either an effect on the constitutive rate of generation of O_2^- or on their ability to release iron from ferritin.

Thus, the autophagic degradation of ferritin most likely accounts for the physiologic origin of the ferric iron pool required for the cell killing by H_2O_2. More broadly, the pool of deferoxamine-chelatable ferric iron is felt to represent iron that moves very transiently in and out of ferritin in the normal process of the uptake and mobilization of ferric iron by all cells. The size of this pool, the kinetics of the passage of iron through it, and its intracellular localization are the concern of current research efforts (*38*).

The Role of Lipid Peroxidation

If it is not detoxified by glutathione peroxidase, H_2O_2 can react with ferrous iron to generate a more potent oxidizing species, such as the •OH. •OH is highly reactive and can interact with cellular targets in ways that may result in lethal cell injury. Several biochemical alterations have been described and, in turn, proposed as bases for the irreversible injury that may follow exposure of cells to partially reduced oxygen species. Attention has focused largely on three cellular targets, namely, the mitochondria, DNA, and the unsaturated fatty acids of membrane phospholipid.

The carbon–hydrogen bonds of a polyunsaturated fatty acid are weakened by an adjacent double bond. Thus, allyic hydrogens are susceptible to abstraction by free radicals with one or more unpaired electrons. Potent oxidizing species, like the •OH, can readily extract such a hydrogen from a membrane fatty acid, a reaction that leaves the carbon bearing a single unpaired electron. The resulting lipid radicals (L•) react with molecular oxygen to form lipid peroxyl radicals (LOO•). LOO• is also a strong oxidizing species and extracts a second allylic hydrogen atom from another methylene carbon. In this manner, the original •OH has now initiated an autocatalytic process that coverts many of the carbons of the fatty acids of membrane phospholipids to hydroperoxides. Such peroxides are unstable and readily fragment to produce a variety of lower molecular weight products. This sequence is termed lipid peroxidation and results in the destruction of the unsaturated fatty acids of membrane phospholipids.

Lipid peroxidation is one of the best known manifestations of oxidative cell injury. Nevertheless, the role that lipid peroxidation plays in the pathogenesis of irreversible cell injury with an acute oxidative stress has been a matter of continued debate (*39–41*). It is difficult to define conditions under which lipid peroxidation is causally related to irreversible cell injury. The association of lipid peroxidation with a particular example of irreversible cell injury does not necessarily imply that the two are causally related. In particular, lipid peroxidation can be a result of the cell injury or an epiphenomenon of the action of the hazard in question. Furthermore, protection by antioxidants may be due to an action other than the inhibition of lipid peroxidation. In particular, the antioxidant might react with the oxidizing species that initiates the lipid peroxidation.

Lipid peroxidation has been largely dismissed as a significant factor in the pathogenesis of lethal cell injury by activated oxygen species (*41–43*). This interpretation of the significance of lipid peroxidation has usually occurred in parallel with the argument for a differing mechanism of cell injury. Such arguments have favored glutathione peroxidase as a toxifying pathway in the metabolism of H_2O_2. Just as there is no role for iron in such a scheme, there is no role for lipid peroxidation.

However, it has been established in one model of oxidative cell injury, namely, the iron-dependent killing of cultured hepatocytes by TBH, that the lethal cell injury is in fact mediated by the peroxidation of membrane lipids (*18*). This conclusion was established by ruling out two of the above three ways that lipid peroxidation may relate to cell death, that is, as a consequence of the cell killing, or as an epiphenomenon of the cell killing, rather than as the cause of the cell killing. Importantly, these three possibilities differ with respect to the effect that an antioxidant would have on the cell killing. If lipid peroxidation simply follows the death of the cells, or is an epiphenomenon that accompanies the primary action of TBH, then an antioxidant would prevent the lipid peroxidation without any effect on the viability of the cells. However, the antioxidants *N,N′*-diphenyl-*p*-phenylenediamine (DPPD) and catechol prevented both the killing of cultured hepatocytes by TBH and the peroxidation of cellular lipids. Thus, if DPPD and catechol act by reacting with lipid radicals (L• or LOO•) to prevent lipid peroxidation, then the peroxidation of cellular lipids would indeed be related to the killing of the hepatocytes.

However, if DPPD or catechol react with *t*-butyl alkoxyl radials, such a conclusion would not be allowed. In this situation, the preservation of cell viability in parallel with an inhibition of lipid peroxidation would be consistent with any of the three possible scenarios outlined above. At concentrations that protected the hepatocytes, DPPD and catechol do not prevent the detection of *t*-butyl alkoxyl radicals (*18,31*). Thus, their protective action is not due to an effect on the formation or detoxification of *t*-butyl alkoxyl radicals. This conclusion allows a causal relationship to be drawn between the appearance of lipid peroxidation and the subsequent death of cultured hepatocytes intoxicated with TBH (*18*).

This causal relationship between the peroxidation of lipids and the killing of cells by TBH suggests that lipid peroxidation is similarly related to the toxicity of activated oxygen species under other conditions. In particular, several situations have been examined in which activated oxygen species are implicated in the genesis of lethal cell injury, including the killing of cultured hepatocytes by H_2O_2 (*21*), allyl alcohol (*44*), diethyl maleate (*45*), dinitrofluorobenzene (45), acetaminophen (*46*), and bromobenzene (*47,48*). In each case, lipid peroxidation is a prominent manifestation of the cell injury. In each case, antioxidants prevent both the peroxidation of lipids and the loss of viability.

Such a conclusion has relevance beyond the killing of cultured cells by an oxidative stress. The mechanism of the killing of rat liver cells in the intact animal by allyl alcohol (*49*), acetaminophen (*50*), and bromobenzene (*51*) closely resembles the action of the same hepatotoxins *in vitro*. In particular, the liver necrosis produced by both bromobenzene and acetaminophen is likely the result of metabolism-dependent oxidative stress and the resultant peroxidation of membrane lipids. In addition, with both bromobenzene (*51*) and acetaminophen (*50*), the covalent binding of their electrophilic metabolites was readily

dissociated from the appearance of liver necrosis by the use of iron chelators, SOD, or antioxidants.

Mitochondria as Target Organelles: Iron-dependent, Nonperoxidative Oxidative Cell Injury

The peroxidation of membrane phospholipids is not the only way that the iron-dependent generation of potent oxidizing species can lethally injure cells. Nonperoxidative mechanisms of lethal cell injury can be readily demonstrated by exposing cells to activated oxygen species in the presence of antioxidants that prevent the peroxidation of cellular lipids (*51–53*). Under such a circumstance, irreversible cell injury develops in the absence of detectable lipid peroxidation.

The circumstances that determine whether a given cell is injured by lipid peroxidation or by a nonperoxidative mechanism are important to note. Acetaminophen kills cultured hepatocytes by peroxidizing membrane lipids (*46*). However, acetaminophen itself is an antioxidant. Thus, as its concentration is raised, lipid peroxidation is inhibited (*46*). However, the cells are still lethally injured by larger doses of acetaminophen, and the cell killing is still mediated by activated oxygen species (*46*). Clearly, the antioxidant activity of any given dose of acetaminophen determines which mechanism will predominate. A similar explanation accounts for the absence of lipid peroxidation with the killing of cultured hepatocytes by menadione (*53*).

Recent evidence suggests that mitochondrial damage is the biochemical basis of the nonperoxidative mechanism of oxidative cell injury (*52*). That the loss of mitochondrial function can lead to the development of irreversible cell injury is, of course, attested to by the effects of ischemia on cells. In this situation, cell killing is correlated with a loss of mitochondrial energization rather than with the depletion of ATP alone (*52,54*). A similar loss of mitochondrial energization occurs in cultured hepatocytes intoxicated with H_2O_2 (*21*), TBH (*52*), or menadione (*19*). Importantly, in each case, the collapse of the mitochondrial membrane potential and the cell killing were iron-dependent (*19,52*). This iron-dependent loss of mitochondrial energization occurred in the absence of lipid peroxidation.

Thus, it is very likely that the nonperoxidative mechanism whereby cells are irreversibly injured by activated oxygen species is similar to the mechanism whereby they are injured in the absence of oxygen, that is, by anoxia. Such a conclusion is supported by the observation that manipulations that modify the toxicity of mitochondrial poisons, similarly modified the killing of cultured hepatocytes by TBH in the presence of an antioxidant (JL Farber, unpublished data).

DNA as the Target Molecule

A number of different lesions in DNA can be produced by such potent oxidizing species as the •OH. Reaction with deoxyribose results in fragmentation with loss of the base and the appearance of a strand break (*55,56*). Alternatively, reaction with thymine produces a variety of lesions removed by repair enzymes that similarly produce single-strand breaks in DNA (*55,56*). Thus, the appearance of single-strand breaks in DNA is a common consequence of the interaction of oxygen radicals with DNA. Single-strand breaks have been reported to accumulate in bacteria and in a variety of mammalian cells upon exposure to oxygen radicals generated by a number of different mechanisms (*55–61*).

In turn, oxidative DNA damage has been implicated in the killing of both bacterial and mammalian cells by oxygen radicals. Mammalian cell lines exposed to H_2O_2 showed a dose-dependent relationship between the accumulation of DNA single-strand breaks and the extent of cell killing (*58*). SOD decreased the number of thymine glycols produced by benzo[*a*]-pyrene in epithelial cells at the same time that it increased cell survival (*60*). The EM9 mutant of the CHO cell line was more sensitive to the toxicity of H_2O_2, a situation that correlated with a decreased ability of the cells to repair lesions in DNA (*57*). Finally, inhibition of poly (ADP-ribose) polymerase, an enzyme that is activated by DNA strand breaks, protected $P388D_1$ cells from the toxicity of H_2O_2 (*61*). This result was interpreted as indicating that the metabolic responses to DNA damage may initiate a sequence of events that result in cell killing (*61*).

Several concerns are raised by these studies of the relationship between oxidative DNA damage and cell killing. First, manipulations that alter the formation or reactivity of oxygen radicals, such as the use of iron chelators or •OH scavengers, did not distinguish between radical-mediated DNA damage and other lesions in the cell that may relate more directly to the loss of viability. Thus, the protective effect of iron chelations against the lethal cell injury occurring with an oxidative stress cannot necessarily be attributed to the protective effect against the accumulation of single-strand breaks in DNA. Clearly, the other chemical effects of the •OH (or alkoxyl radical in the case of TBH) are also prevented by iron chelation, as well as by radial scavengers. Thus, in order to specifically assess the role of DNA damage in the cell killing by an oxidative stress, it is necessary to manipulate the sensitivity of the cell to activated oxygen species by means that act on events that occur after the formation of the •OH.

When such a concern is observed, it can be shown that damage to DNA can be dissociated from the mechanisms of the oxidative killing of cultured hepatocytes (*31*). Stated differently, the killing of cultured hepatocytes by an acute oxidative stress does not necessarily result from the evident damage to the DNA. This was shown by manipulating the toxicity of TBH at a point after the iron-dependent formation of the *t*-butyl alkoxyl radical, the species that is believed to be responsible for initiating the damage to DNA (*31*).

The appearance of single-strand breaks in the DNA of hepatocytes intoxicated with TBH required a cellular source of ferric iron (*31*). The pretreatment of the cells with deferoxamine protected the hepatocytes from the accumulation of single-strand breaks (*31*). In a number of other models of oxidative stress, DNA damage has similarly been shown to depend on a source of ferric iron (*55,59,62,63*).

The antioxidant DPPD acts at a point distal to the formation of the *t*-butyl alkoxyl radical to inhibit lipid peroxidation (*18,31*). Thus, the hepatocytes are protected from lethal injury at a step subsequent to the formation of the radical that damages DNA. DNA damage still occurred in hepatocytes exposed to TBH in the presence of DPPD, despite the fact that the cells did not die (*31*). Similarly, acidification of the culture medium prevented the cell killing by TBH without any effect on the extent of the DNA damage (*31*).

Another interpretation of the effects of both DPPD and extracellular acidosis would argue that both manipulations act to prevent some mechanism that couples DNA damage to lethal cell injury. However, such a conclusion would have to postulate that the presumed coupling mechanism acts to initiate lipid peroxidation. Evidence for such a mechanism is lacking in hepatocytes.

A mechanism coupling single-strand breaks in DNA to lethal cell injury has

been proposed to operate in the killing of $P388D_1$ cells, a murine macrophage-like tumor cell line, exposed to H_2O_2 (*61*). Inhibitors of poly(ADP-ribose) polymerase, a nuclear enzyme activated under various conditions of DNA damage and repair, prevented the killing of $P388D_1$ cells by H_2O_2. Single-strand breaks in DNA activated poly(ADP-ribose) polymerase in the attempt to repair this damage (*64*). The consequent binding of ADP-ribose to proteins was responsible for a depletion of cellular stores of NAD and ATP (*61,64*). In turn, the latter is argued to lead to an accumulation of intracellular Ca^{2+} ions, actin polymerization, and finally, cell death (*61*).

There is no evidence for a role of activation of poly(ADP-ribose) polymerase in the killing of cultured hepatocytes by an oxidative stress (*65,66*). By contrast, the killing of L929 mouse fibroblasts by H_2O_2 was prevented by inhibitors of poly(ADP-ribose) polymerase (*66*). Thus, the role of DNA damage and activation of poly(ADP-ribose) polymerase plays in oxidative cell killing depends on the cell type. The differing consequences of oxidative damage to DNA in fibroblasts and hepatocytes can be related to another important feature distinguishing these cells. L929 fibroblasts are proliferating cells, whereas hepatocytes are resting cells, which do not normally divide. Single-strand breaks inhibit DNA replication and thereby cellular proliferation. Thus, it might be expected that a proliferating cell, such as the L929 fibroblast, would rapidly repair any damage to its DNA, an activity that requires poly(ADP-ribose) polymerase. By contrast, hepatocytes would be expected to be less attentive to single-strand breaks, since they are not usually replicating their DNA.

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